Social experience during adolescence in female rats increases 50 kHz ultrasonic vocalisations in adulthood, without affecting anxiety-like behaviour

Running Head: Adolescent social experience enhances female adult rat 50kHz call rate

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Abstract

Adolescents are highly motivated to engage in social interactions, and researchers have hypothesised that positive social relationships during adolescence can have long-term, beneficial effects on stress reactivity and mental wellbeing. Studies of laboratory rodents provide the opportunity to investigate the relationship between early social experiences and later behavioural and physiological responses to stressors. In this study, female Lister-hooded rats (N=12 per group) were either i) provided with short, daily encounters (10 minutes per day) with a novel partner during mid-adolescence (postnatal day 34-45; 'social experience', SE, subjects) or ii) underwent the same protocol with a familiar cagemate during mid-adolescence ('control experience', CE, subjects), or iii) were left undisturbed in the homecage (non-handled 'control', C, subjects). When tested in adulthood, the groups did not differ in behavioural responses to novel environments (elevated plus-maze, open field, light-dark box) or in behavioural and physiological (urinary corticosterone) responses to novel social partners. However, SE females emitted significantly more 50kHz ultrasonic vocalisations than control subjects both before and after social separation from a familiar social partner, which is consistent with previous findings in male rats. Thus, enhanced adolescent social experience appears to have long-term effects on vocal communication and could potentially modulate adult social relationships.

Keywords: adolescence, social novelty, neophobia, ultrasonic vocalisations, glucocorticoids

1. Introduction

Adolescents exhibit a strong motivation to engage in social interactions with peers (Nelson *et al*., 2016; Trezza *et al*., 2011). Although some social interactions, such as peer rejection, can be a major source of stress during this stage of life (Conley & Rudolf, 2009), positive social experiences can act as a buffer against the deleterious effects of these stressors (Gunnar & Hostinar, 2015). These positive social interactions could have long-term, as well as immediate, impacts on stress reactivity and emotionality. For instance, strong adolescent friendship networks amongst human beings have been linked with lower anxiety and depression symptoms in early adulthood (Narr *et al*., 2017). During adolescence, a number of brain areas undergo significant developmental change, including regions involved in emotional and social processing, such as the amygdala and prefrontal cortex (Tottenham & Galván, 2016). The hypothalamic-pituitary-adrenal (HPA) axis, a neuroendocrine system that mediates responses to possible threats, also exhibits plasticity during adolescence (Romeo *et al*., 2016). Adolescent social contact may improve emotional wellbeing via enhanced prefrontal cortex functioning (Bell *et al.,* 2010) and diminished HPA axis reactivity (Terranova *et al.,* 1999), whilst adolescent social stress may impair wellbeing by modulating amygdala development (Tsai *et al.,* 2014) and potentiating HPA axis activity (Isgor *et al.,* 2004). Adolescent social interactions, via a range of neural and physiological processes, are therefore able to have long-term effects on affective and social responses (Sachser *et al*., 2013).

Research on laboratory rodents has investigated how social experiences during adolescence influence later behavioural, neural and endocrine responses to stressors (McCormick *et al*., 2015). In many species, adolescents exhibit high levels of social play (e.g. male rats: Klein *et al*., 2010; mice: Terranova *et al*., 1993). Social play is rewarding for adolescent rodents (Trezza *et al*., 2011) and enhances the development of adult social skills and emotional competencies (Himmler *et al*., 2016; Pellis & Pellis, 2017). Depriving adolescent rodents of the opportunity to interact socially, via isolation housing, has been shown to have long-term dampening effects on adult social

interactions and to increase anxiety-like behaviour and disrupt fear extinction (e.g., male rats: Seffer *at al*., 2015; Skelly *et al*., 2015; van den Berg *et al*., 1999; see Burke *et al*., 2017a). Adolescent social isolation has also been reported to have effects on later HPA axis functioning, such as elevated circulating glucocorticoid concentration (e.g., rats: Lukkes *et al*., 2009; Pisu *et al.*, 2106; McCormick *et al*., 2015). Diminished social contact during adolescence may result in greater HPA axis activity; for instance, fewer opportunities for play has been linked to diminished functioning of the medial prefrontal cortex (Bell *et al.,* 2010), an area that typically exerts an inhibitory effect on the HPA axis (Herman *et al.,* 2003).

Additional evidence for the long-term effects of adolescent social experience is provided by the literature on environmental enrichment, where group-housed animals are commonly described as experiencing 'social enrichment' (Girbovan & Plamondon, 2013; Simpson & Kelly, 2011). This research has shown that rodents housed with three or more peers during adolescence exhibit reduced anxiety-like behaviour and enhanced social behaviour in adulthood compared to controls (e.g., rats: Brenes *et al*., 2016; Harati *et al*., 2013; Peña *et al*., 2009). Social and physical enrichment potentially acts as a mild stressor that produces an adult phenotype that is better able to cope with subsequent novel situations (Crofton *et al*., 2015), and this hypothesis is supported by evidence that adolescent social enrichment reduces HPA activation when exploring a novel environment in adulthood (e.g., male guinea pigs: Lürzel *et al*., 2010; 2011; rats: Peña *et al*., 2009). Adolescent isolation housing and social enrichment therefore do not just shape social behavior, but also appear to have substantial effects on anxiety-like responses and stress physiology in later life.

Adolescent social instability has also been used to examine how adverse experiences during early life can have long-term detrimental effects (McCormick *et al*., 2015). A standard social instability paradigm has been developed for adolescent rodents, which involves a combination of repeated daily isolation (e.g., 1 hour) followed by pairing with a new social partner

each day (e.g., postnatal day, PND, 30-45; McCormick, 2010). Adolescent social instability has been shown to have adverse outcomes, such as increased anxiety-like responses in novel environments and reduced social interactions with unfamiliar peers in adulthood (e.g., mice: Caruso *et al*., 2017; Schmidt *et al*., 2007; male rats: Green *et al*., 2013; Hodges *et al*., 2017; see McCormick & Green, 2013; McCormick *et al*., 2015). However, evidence for long-term effects of adolescent social instability on HPA functioning has been inconsistent, with adult rodents exposed to social instability during adolescence exhibiting blunted, similar or raised corticosterone levels relative to control subjects (e.g., mice: Caruso *et al*., 2017; Schmidt *et al*., 2007; rats: Mathews *et al*., 2008; McCormick *et al*., 2008; see Brown & Spencer, 2013).

The long-term effects of adolescent social experience are likely influenced by a range of factors (Hodges *et al*., 2018), including whether the subjects are male or female. Most studies of adolescent social experience have used male rodents (Burke *et al*., 2017a), even though, in human beings, a female-biased prevalence in affective disorders emerges during this stage of life (Kessler *et al*., 2005). Adolescent social interactions can modulate development in female rats (Bell *et al.,* 2010; Schneider *et al.,* 2016), but the long-term effects of manipulating social experience during adolescence on anxiety-like behaviour often differ between female and male rodents, depending on the experimental paradigm; for instance, unlike males, female rats singly housed during adolescence do not differ from controls in their anxiety-like behaviour in adulthood (e.g., Butler *et al*., 2014; Jahng *et al.,* 2012; Weintraub *et al*., 2010) and adolescent social instability has a stronger negative impact on females than males (e.g., Bourke & Neigh, 2011; Caruso *et al*., 2018; Weathington *et al*., 2012). Sex differences in response to adolescent single housing and social instability emphasise that adolescent social experiences are not always adverse, but may have beneficial or negligible effects depending on whether male or female animals are tested.

Male rodents that have undergone social isolation or social instability during adolescence exhibit reduced testosterone levels and impaired sexual behaviour in adulthood compared to

control subjects (e.g., McCormick *et al*., 2013; Ward & Reed, 1985). In addition, male rats that have experienced adolescent social instability are less preferred as a sexual partner (McCormick *et al*., 2017), spend less time interacting with novel same-sex conspecifics (Green *et al*., 2013) and exhibit more aggressive behaviour towards cage-mates during food competition (e.g., Cumming *et al.*, 2014), compared to control subjects. These findings are consistent with the hypothesis that lack of appropriate social experience during adolescence impairs later social competence. Vocal communication is an important component of social interactions (Wöhr *et al.,* 2015). Male rodents, for example, can emit 50 kHz ultrasonic vocalisations (USVs) in order to solicit social contact and socio-sexual interactions (Brudzynski, 2005) and prevent playful interactions from escalating into aggression (Burke *et al.,* 2017b). Adolescent rats also emit 50kHz vocalisations during play and such calls can, in turn, facilitate further playful interactions (Burke *et al.,* 2018). Consistent with the notion that more adolescent social experiences can enhance social competency, male rats that are group housed during adolescence emit more 50kHz ultrasonic vocalisations (USVs) in response to female partners and exhibit greater approach behaviour to playbacks of 50kHz USVs when in adulthood compared to adult rats that were single housed in adolescence (Inagaki *et al*., 2013; Seffer *et al*., 2015; see Wöhr et *al*., 2015). In comparison, relatively little is known about the effects of adolescent social experience on later sexual and social competence, including vocal communication, in female rodents (Burke *et al*., 2017a).

The aim of this study was to examine the long-term effects of enhanced social experience during adolescence on later anxiety-like behaviour and social behaviour in female rats. Our design used short-term social encounters that involved pairing female rats with a novel same-sex partner for 10 minutes once per day over a 12-day period (PND 34-45, i.e., mid-adolescence; Lynn & Brown, 2009). Such short-term encounters have been widely used in adult rats to quantify social exploration (File & Seth, 2003), and the adolescent age range in which rats in the current study were manipulated are comparable to previous research investigating adolescent social instability

(e.g. McCormick *et al.,* 2010). In comparison to social instability tests in which rats are briefly single housed then pair-housed with a new partner on each subsequent day (e.g. McCormick *et al.,* 2010), adolescent rats in the current study were not single housed and had a familiar home-cage partner to return to each day. Short-term social encounters during adolescence may therefore be expected to act as a form of social enrichment that potentially enhances social competencies and diminishes anxiety-like behaviour when in adulthood, as occurs in response to other forms of social enrichment (Girbovan & Plamondon, 2013; Simpson & Kelly, 2011). During adolescence the amount of time spent interacting with a novel or familiar social partner was recorded, and, in adulthood, we recorded behavioural responses to novel environments (elevated plus-maze and light-dark box), social preference for a novel male conspecific, and USV production in response to separation from a same-sex cage-mate. In addition, urinary corticosterone levels were measured following social separation to examine the effects of adolescent social experience on HPA activity, given that environmental enrichment during adolescence has previously been shown to dampen the HPA axis (Morley-Fletcher *et al.,* 2003). Female rats experiencing enhanced social experience were predicted to exhibit lower levels of anxiety-like behaviour, lower corticosterone production, and higher levels of social engagement than controls when in adulthood.

2. Methods

2.1. Ethical statement

Ethical guidelines as set out in the Principles of Laboratory Animal Care (NIH, Publication No. 85–23, revised 1985) and the UK Home Office Animals (Scientific Procedures) Act 1986 were adhered to under Project Licence 60/4354 and Personal Licence IDFA58352.

2.2. Animals and housing

The subjects were 36 female Lister-hooded rats, bred in-house from nine litters (stock animals from Envigo, UK). An additional 14 females and 6 males from these litters were used as social

interaction partners ('playmates'). All pups were reared by their mothers in plastic and wire-mesh cages (52 x 40 x 26 cm, length x depth x height), then weaned on PND 26 into same-sex sibling pairs, which were housed in plastic and wire-mesh cages (45cm x 28cm x 21cm cages, length x depth x height). All animals had *ad libitum* access to pellet food (DBM Food Hygiene Supplies Ltd, Scotland) and water, and the cages were located in a single holding room on a 12:12 light-dark cycle (lights on at 07:00) with regulated temperature (20±1°) and humidity (55±5%). All subjects were weighed once per week during development, including at the start and end of the adolescent social manipulation, to assess whether all rats were growing at comparable rates.

2.3. Experimental design

i) Adolescent social experience

The three sets of female subjects (N=12 per condition, housed as 6 pairs) differed in the amount of social experience and experimenter handling during adolescence, as follows: i) 'social experience' (SE) subjects were provided with the opportunity to interact socially with a similar-aged (±1 day) female conspecific ('playmate') in an arena for 10 minutes per day for a 12-day period during adolescence (PND 34-45) (i.e., each SE subject interacted with 12 novel partners in total); ii) 'control experience' (CE) subjects were placed into an arena with their familiar cage-mate for 10 minutes per day for the same 12-day period; and iii) non-handled 'control' (C) subjects were left undisturbed in their home cages during this period. Rats from each litter were distributed across the conditions, with no more than two individuals from a litter used in the same condition, and 'playmates' were not from the same litter as the subject undergoing testing.

Two identical test arenas were used for the social interaction sessions. Each arena consisted of an open-topped box constructed from perspex (48cm x 47cm x 44 cm, length x height x depth) with black cardboard on the outside of the walls and sawdust on the floor. The arenas were located adjacent to one another in a testing room and were surrounded by a black curtain. For the SE

condition, a pair of subjects and a pair of playmates were transported separately to the testing room, and one of the subjects was placed into each of the arenas, followed by a playmate. For CE subjects, a pair of subjects was transported to the testing room, and both animals were placed into one arena. At the end of each session (i.e., after 10 minutes had elapsed), all animals were returned to the home-cages, and the apparatus was cleaned with 70% alcohol solution.

During social interaction sessions, behaviour was observed via a ceiling-mounted camera projecting to a computer monitor, and behavioural data were entered by an observer using an in-house software programme. The arena was visually divided into four equal quadrants, and the following behavioural measures were calculated: i) *duration of time both rats were in the same quadrant* (seconds), ii) *number of play behaviour bouts* (nape attacks, boxing, pinning or evasion), and iii) *number of investigative sniffs* (facial, flank and anogenital sniff) (definitions follow Cirulli *et al*., 1996 and Klein *et al*., 2010). As the ceiling-mounted camera was located above only one arena, both sets of animals could not be observed in the SE sessions; therefore, behavioural data were collected for only one pair of animals (i.e. data were collected of six out of twelve sessions for each SE subject), and the same number of sessions were therefore observed for CE pairs. C pairs were left in their home cages during adolescence and their social behavior were therefore not recorded.

ii) Adulthood behavioural testing

In adulthood, all subjects underwent three periods of testing. During the first period (PND 96-111), the subjects were tested in: a) an elevated plus-maze, b) a light-dark box, and c) a social novelty task (see '*Response to novelty*'). The order of testing was counter-balanced across conditions, and each subject was tested on one task per week (i.e., on PND 96/97, PND 103/104 and PND 110-111). The second period (PND 122-132) involved assessment of urinary corticosterone concentrations following a 3-hour period of housing in a novel home cage with an

unfamiliar female partner or familiar partner (i.e., cage-mate), with all subjects experiencing both conditions on consecutive days and with the order counterbalanced across conditions (see '*CORT response to social interactions*'). The third period of testing (PND 156-169) involved a social separation task, in which ultrasonic vocalisations were recorded before, during and after separation from a familiar partner (i.e., cagemate) (see '*Ultrasonic vocalisations before, during, and after social separation'*).

2.4. Apparatus and measures

i) Response to novelty

All subjects were tested individually. In all tests, the apparatus was surrounded by a black curtain and cleaned with 70% alcohol solution after each animal, and behavioural data were recorded via a ceiling-mounted camera.

a) The *elevated-plus maze (EPM)* consisted of a wooden plus-shaped maze that was raised 56cm off the floor using a metal frame. The four wooden arms (51cm x 11cm, length x width) extended out from a central area (11cm x 11 cm). Two of the arms had walls ('closed' arms; 40cm height), and two did not have walls ('open' arms). At the start of the test, the subject was placed into the central area, facing an open arm, and each test lasted 5 mins. The animal was recorded as entering a new area when all four paws crossed onto a new arm or into the central area, and the following scores were calculated: i) *total number of entries into closed and open arms*, and ii) *percentage of time spent on the open and closed arms*.

b) The *light-dark box (LDB)* consisted of a perspex arena (119cm x 44cm x 47 cm; length x width x height), which was divided into two compartments using an opaque plastic divider with an opening at floor level (11cm diameter archway). The smaller, 'dark' compartment (49cm x 44cm x 47cm cm; length x width x height) had a wooden lid and black card on the exterior walls, while the larger, 'light' compartment (70cm x 44cm x 47cm; length x width x height) was lit from above

by an artificial white-light source (150lux) and had white cards on the exterior walls. At the start of the test, the subject was placed into the dark compartment, and the test lasted for 5 mins. The animal was recorded as entering a new area when all four paws crossed into another compartment, and the following scores were calculated: (i) *latency to enter the light compartment (seconds)*, ii) *number of entries into the light compartment*, and iii) *percentage of time spent in the light compartment*.

c) The *social novelty (SN) task* was conducted in a perspex arena (119cm x 44cm x 47cm (length x width x height) with black card on the exterior walls. Two perforated transparent perspex boxes (24cm x 21cm x 46cm; length x width x height) were placed into opposite corners of the arena. During testing, one box ('social box') contained an unfamiliar male rat (i.e., non-siblings), while the other box ('object box') contained a novel object (five objects were used, e.g., a glass jar filled with grey stones and a blue plastic bottle, which were counterbalanced across conditions). At the start of the test, which lasted 5 mins, the subject was placed into the central area. Animals were considered to be in contact with the stimulus box when touching the box with head, body, or paws, and the following scores were calculated: i) *number of contacts with the social box and object box,* and ii) *percentage of time in contact with the social box and object box* (seconds).

ii) CORT response to social interaction

A pair of subjects was transported to a small testing room, and a urine sample was collected from each subject by holding the animal over a clean, plastic cagebase (max. 10 mins). A minimum of 50 µl of urine was collected from the floor using a pipette (i.e. pre-interaction sample), then stored at -20°C until assayed. The subjects were then moved to another testing room and pair-housed with either a familiar (i.e. cagemate) or unfamiliar same-aged female rat (cages measured 39cm x 57cm x 26cm, length x width x height, and contained *ad libitum* pellets and water). After three hours, the rats were moved back to the original testing room, and a second urine sample was

collected (i.e. post-interaction sample), and all animals were then returned to home cages. Three hours is sufficient for determining changes in urinary CORT concentration (Bamberg *et al.,* 2001), and is sufficiently long to induce a physiological stress response (Terranova *et al.,* 1999). All rats underwent urine sampling under both social conditions (i.e. housed with familiar or unfamiliar rats).

Creatinine concentrations in the urine samples were quantified using an enzyme immunoassay kit (Creatinine (urinary) Colorimetric Assay, Cayman Chemical, USA). 5µl urine samples were diluted to a concentration of 1:20, and samples were run in duplicate across two plates, which were read on a Biochrom Anthos 2010 Microplate Reader (Biochrom Ltd., UK). Intra-plate coefficients of variation were 9.74% and 11.52%, and inter-plate coefficient of variation was 10.63%. CORT concentrations were quantified using radioimmunoassay, following a previously described protocol (Spencer *et al.,* 2009). All samples were run in duplicate across two assays. 50% binding (ng/ml) for each assay was 0.71 and 0.75. Intra-assay coefficients of variation were 14.35% and 11.33%, and inter-assay coefficient of variation was 12.84%. In order to scale CORT concentrations for urine concentration, urinary CORT concentration was expressed as a ratio between CORT and creatinine concentrations, i.e. 10^{-5} CORT (ng/ml)/creatinine (mg/dl).

iii) Ultrasonic vocalisations before, during, and after social separation

The apparatus consisted of a perspex arena, which was divided into two compartments (each measuring 32cm x 44cm x 47cm, length x width x height) using a perforated transparent barrier that allowed visual, olfactory and vocal communication. Two ultrasound microphones (CM16/CMPA, Avisoft Bioacoustics, Germany) were positioned above the arena, one on either side of the barrier. The analogue microphone output was digitized using an Edirol FA101 sound card (Roland Corp., Japan; 192kHz sampling rate in 24-bit format), which was operated using open source software (Audacity, version 2.0.5), and stored as .wav files. Ultrasonic vocalisations

(USVs) were visualised as spectrograms in Audacity and categorised as 50kHz (frequency range 30-80kHz) or 22kHz USVs (frequency range 20-25kHz) (based on definitions in Wright *et al.,* 2010). Only 50 kHz calls are presented, as fewer than 10% of rats emitted 22kHz UVSs.

The social separation task consisted of three phases: pre-separation, separation, and reunion. During pre-separation, a pair of rats that were from the same home cage were placed into the arena, with one rat in each compartment, for five minutes, and USVs were recorded. One of the subjects was then placed into a cage (39cm x 57cm x 26cm, length x width x height, with *ad libitum* pellets and water) in the testing room, and the other subject was placed into a similar cage in another testing room. The microphones were moved so that one was suspended above each cage, and USVs were recorded for the first five minutes of a 60 minute separation period. The subjects were then placed back into the original arena, with one animal per compartment with a microphone, and USVs were recorded for a 5 minute period, after which the subjects were returned to the home cage. To control for any potential effects of being moved to a different room, each subject was tested twice (once remaining in the original testing room and once being moved to the other room), with seven days between the tests, and test order was counter-balanced across conditions.

2.6. Data analysis

All analyses were conducted using R (version 1.1.463). Adolescent social behaviour, body weight, EPM and SN data were analysed using linear mixed models (LMMs), with litter size entered as a random factor in all models and repeated measures used where appropriate (e.g., session number in the adolescent social interaction data and arm type in the EPM data), using the 'nlme' package (Pinheiro *et al.,* 2018). LDB data were analysed using a multivariate analysis of variance. Change in urinary CORT/creatinine concentration (i.e. pre-interaction minus post-interaction CORT/creatinine concentration) was calculated to determine the change in CORT in response to

familiar or unfamiliar rats, and analysed using a LMM with partner adolescent condition and litter size entered as random factors. Pre-interaction 10^{-5} CORT (ng/ml)/creatinine (mg/dl) did not differ with adolescent condition (general linear model: $F_{2,69} = 0.236$, p = 0.791) and was entered as a co-variate in the model. Ultrasonic calls were analysed using a generalized linear model with data fitted to a gamma error distribution using the 'glm2' package (Marschner, 2011). Prior to analyses, residuals were checked for normality (Shapiro-Wilk) and variables that were positive skewed were transformed (square-root or log10) to achieve normality or analysed using generalized linear models. An alpha value of $p<0.05$ was used throughout, with significant main effects and interactions further explored using Bonferroni *post-hoc* tests. Cohen's d was calculated as a measure of effect size for all significant *post-hoc* pairwise comparisons. All data are presented as means $\pm$ one standard error. Data available on request from the authors.

3. Results

3.1. Body weight

The subjects gained weight between the start and end of the adolescent social manipulation (age: $F_{1,33} = 9630.55$, $p < 0.001$, d = 11.391), and body weight did not differ on average between the conditions (condition: $F_{2,33} = 3.165$, p = 0.055; age x condition: $F_{2,33} = 0.168$, p = 0.846; PND 32: SE = 56.4$\pm$1.81g; CE = 60.8$\pm$1.76g; C = 55.1$\pm$1.66g; PND 45: SE: 150.2$\pm$2.41g; CE: 153.0$\pm$3.02g; C = 147.0$\pm$2.83g).

3.2. Adolescent social interactions

The results confirmed that SE females spent a greater proportion of time in the same quadrant as their social partner than did CE females ($F_{1,17} = 23.554$, $p < 0.001$, d = 1.26; SE = 54.80±0.91%; CE = 44.70±1.40%), an effect that did not differ with test session (session: $F_{5,79} = 0.138$, p = 0.714; session x condition: $F_{5,79} = 0.977$, p = 0.326). SE pairs also engaged in more play behaviour than CE pairs ($F_{1,17} = 14.653$, $p < 0.001$; SE = 5.56±0.53 play interactions per session; CE = 2.31±0.37

play interactions per session), and this difference depended upon the test session ($F_{5,79}$ = 9.281, p < 0.001; **Figure 1**): the difference between conditions was only apparent in sessions 4, 5 and 6 (session 4: p = 0.02, d = 1.154; session 5: p < 0.001, d = 2.144; session 6: p < 0.001, d = 1.888), and the main effect of session was also significant ($F_{5,79}$ = 6.685, p < 0.001). SE pairs also engaged in more sniffing behaviour than CE pairs ($F_{1,17}$ = 83.929, p < 0.001, d = 1.881; SE = 20.67±0.72 sniffs per session; CE = 10.11±0.73 sniffs per session), which did not depend upon test session (session: $F_{5,79}$ = 1.481, p = 0.205; session x condition: $F_{5,79}$ = 1.503, p = 0.198).

3.3. Adult behavioural responses to unfamiliar environments

Elevated-plus maze. Rats entered the open arms more than the closed arms ($F_{2,33}$ = 102.607, p < 0.001; **Figure 2a**) and spent a greater proportion of time on the open arms than the closed arms ($F_{1,33}$ = 67.925, p < 0.001, d = 2.542; **Figure 2b**). However, number of arm entries and proportion of time spent on each arm did not differ with adolescent condition (entries: $F_{2,33}$ = 2.064, p = 0.143; time: $F_{2,33}$ = 0.350, p = 0.707), and the interaction between condition and arm type was also not significant for either variable (entries: $F_{2,33}$ = 1.150, p = 0.329; time: $F_{2,33}$ = 0.368, p = 0.695).

Light-dark box. Regardless of adolescent condition, rats entered the light compartment after a similar duration of time ($F_{2,29}$ = 0.102, p = 0.903), entered the light compartment a similar number of times ($F_{2,29}$ = 0.463, p = 0.634), and spent comparable durations of time in the light compartment ($F_{2,29}$ = 0.052, p = 0.949) (**Table 1**).

Social novelty. Subjects were in contact with the social box more than the object box ($F_{1,32}$ = 90.174, p < 0.001; **Figure 3a**) and spent a greater proportion of time in contact with the social box than the object box ($F_{1,32}$ = 151.581, p < 0.001, d = 3.041; **Figure 3b**). Number of times, and duration of time, in contact with a stimulus box did not differ with adolescent condition (contacts: $F_{2,32}$ = 1.708, p = 0.197; duration: $F_{2,32}$ = 3.139, p = 0.057), and the interaction between condition

and stimulus box type was not significant for either variable (contacts: $F_{2,32} = 0.834$, p = 0.444; duration: $F_{2,32} = 0.686$, p = 0.511).

3.4. Corticosterone response to familiar and unfamiliar conspecifics

Change in urinary CORT/creatinine ratio did not differ with adolescent condition or with partner familiarity (condition: $F_{2,34} = 0.534$, p = 0.470; partner familiarity: $F_{1,34} = 0.426$, p = 0.512; condition x partner familiarity: $F_{2,34} = 1.606$, p = 0.214; **Table 2**).

3.5. USVs before, during, and after social separation

The total number of 50kHz USVs emitted by subjects depended on task phase ($F_{2,91} = 49.795$, p < 0.001; pre-separation v separation: p < 0.001; pre-separation v reunion: p < 0.001; separation v reunion: p = 0.002) and adolescent condition ($F_{2,91} = 4.417$, p = 0.015), and the interaction between task phase and condition was also significant ($F_{4,91} = 4.181$, p = 0.004; **Figure 4**). Post-hoc analyses revealed that, during pre-separation, SE subjects emitted more calls, on average, than both groups of controls (C vs. SE: p = 0.007, d = 0.986; CE vs. SE: p = 0.008, d = 1.02; C vs. CE: p = 0.825). The number of 50kHz USVs produced during separation did not differ between conditions (C vs. CE, p = 0.495; C vs. SE, p = 0.4296; CE vs. SE, p = 0.300). During reunion, SE subjects emitted more USVs, on average, than C subjects (p = 0.013, d = 0.832) but not CE subjects (p = 0.354), and C and CE females emitted similar number of USVs (p = 0.162).

4. Discussion

In this study, female rats that experienced enhanced levels of social interaction during adolescence did not differ from control females in their responses to novel environments or novel, opposite-sex partners during adulthood. In addition, no differences were found between groups in urinary corticosterone levels following social separation from a familiar, same-sex partner. These findings suggest that, in contrast to adolescent social instability procedures that involve repeated rehousing with novel conspecifics (McCormick *et al*., 2015), short-term social encounters during

adolescence do not appear to have long-term effects on behavioural responses to novel environments and opposite-sex partners, or urinary corticosterone levels, in female rats. However, females that had enhanced social experience during adolescence produced more 50kHz USVs than controls, both before social separation and following reunion with a familiar partner. 50kHz USVs in rats have an important role in co-ordinating and enhancing affiliative and socio-sexual interactions (e.g. Burke *et al.,* 2017c; Inagaki *et al.,* 2013). Our finding that adolescent social stimulation modulates patterns of vocal communication in adulthood in female rats is consistent with a previous study of male rats (Inagaki *et al*., 2013) and potentially reflects long-term effects on social competence.

In our design, SE subjects were placed into an arena with an unfamiliar female for 10 mins per day for a 12-day period of mid-adolescence (PND 34-45), while CE subjects were placed into the arena with their cagemate. Previous studies have reported that adolescent rats exhibit more social play behaviour with novel than familiar partners (e.g., Cirulli *et al*., 1996; Hodges & McCormick, 2015), and, in line with these studies, SE subjects interacted with their social partners at a higher frequency than did CE subjects during these sessions, both in terms of social play and time spent in physical proximity. The difference in frequency of social play between SE and CE subjects was only observed during the later sessions, corresponding to the second half of the exposure days, which potentially reflects a habituation effect to the arena, although other studies have reported that adolescent rats interact more with novel than familiar partners during one-off sessions (Cirulli *et al*., 1996; Hodges & McCormick, 2015; Reinhart *et al.,* 2006). Regardless of potential habituation effects, these data confirm that the experimental design successfully resulted in adolescent SE subjects interacting with novel partners.

In adulthood, SE subjects did not differ from control subjects in terms of response to novel environments (i.e., EPM and LDB). These findings contrast with previous studies that report an increase in adult anxiety-like behaviour in response to adolescent social instability that involves

single housing then pairing adolescent subjects with a new social partner each day (e.g., less time spent on the open arms of the EPM: Caruso *et al*., 2018; Schmidt *et al*., 2007, but see McCormick *et al*., 2008). Our results suggest that enhanced social contact does not have significant long-term effects on the willingness of female subjects to explore potentially aversive areas of novel environments. Whether our experimental design would produce long-term effects in male rats remains to be tested. One reason for the absence of effects could be that the social experiences across all conditions provide sufficient social stimulation for the typical behavioural responses to novelty to develop. Adolescent rats experience close contact with siblings (e.g. Thiels *et al.,* 1990) and occasional interactions with unfamiliar rats when living in colonies (e.g. Calhoun, 1962). Perhaps only social interactions sufficiently above or below these typical experiences, such as social instability or single housing, modify adult behavioural responses to unfamiliar stimuli. A sufficiently high number and duration of novel social interactions during adolescence are needed to modulate adult novelty responses in guinea pigs (Lürzel *et al.,* 2010; 2011), and such effects could also be present in rats.

Adult SE subjects also did not differ from adult control females in the amount of time spent in contact with a transparent barrier that separated the subject from a male conspecific. Although SE subjects spent the highest average amount of time in contact with this barrier, the difference between groups of females was not significant. For all groups, the absolute amount of time spent next to the barrier with the male was very similar to a previous study using the same apparatus in which the position of the two sexes was reversed (Brown *et al*., 2015), suggesting that female rats are as motivated as males to investigate an opposite-sex conspecific in this task. Previous studies have shown that adult female and male rats that have been group-housed during adolescence engage in more social exploration with same- and opposite-sex rats than do rats that have been pair-housed or single-housed during this stage of life (e.g., Brenes *et al*., 2016; Molenda-Figueira *et al*., 2017; Peña *et al*., 2009), while male rats exposed to adolescent social instability exhibit

impaired social interactions with same-sex and opposite-sex peers in adulthood (Green *et al*., 2013; McCormick *et al*., 2013; Ward & Reed, 1985). In the current study, females were not provided with the opportunity to interact directly with males and oestrous cycle stage was not recorded, so, in the future, direct social interactions at specific stages of fertility could be examined to determine whether our adolescent social manipulation enhances social exploration in later-life and under which reproductive states.

The HPA axis is known to undergo considerable development during adolescence and is sensitive to a range of both stressful and non-stressful events during this stage of life (Romeo *et al*., 2016). Adolescent rodents that have experienced social isolation exhibit greater glucocorticoid secretion than group-housed animals in response to stressors in adulthood (e.g., Lukkes *et al*., 2009; Weintraub *et al*., 2010), while adolescent social enrichment decreases later glucocorticoid secretion (e.g., Peña *et al*., 2009). In the current study, urine samples were collected from subjects before and after being paired with an unfamiliar or familiar female for three hours. No differences were found between SE, CE and C females in terms of either absolute corticosterone levels or the change in corticosterone from baseline to post-housing. In addition, no overall effect of partner familiarity was found, in contrast to studies of male rats reporting higher circulating corticosterone levels in males that have been housed with an unfamiliar conspecific compared to those paired with a familiar partner (Caruso *et al*., 2014; Hodges *et al*., 2014); these studies measured serum and faecal corticosterone levels, which potentially reflected HPA functioning more accurately than the urine sampling used in the current study.

In the final test, 50kHz USVs were recorded from subjects immediately prior to one hour of separation from the cage mate, as well as during the first five minutes of the separation period and after being reunited with the partner. SE females produced higher average numbers of 50kHz USVs than CE and C subjects during the pre-separation period, as well as higher average numbers of 50kHz USVs than C subjects during reunion. CE subjects exhibited intermediate levels of USVs

during reunion relative to the other two groups, which suggests that the handling experienced by these control subjects potentially had subtle long-term effects, but this hypothesis would require further investigation. For all subjects combined, the levels of 50kHz USV production were highest during separation, in line with previous evidence that social separation elicits USV production in adult rats (e.g., Wöhr *et al*., 2008). Differences between the groups in pre-separation 50kHz call rate could reflect different affective responses to the novel testing environment (Knutson *et al.,* 2002); however, no behavioural differences between groups were found in the novel environment tasks. Instead, 50kHz USVs are likely reflect differences in sociality, as these calls have an important communicative role in rats (Wöhr *et al*., 2015). 50 kHz USVs are considered to reflect social motivation (e.g., Börner *et al*., 2016) and male rats that have been singly housed during adolescence have been reported to display avoidance behaviour in response to playbacks of 50kHz USVs (Seffer *et al*., 2015). 50kHz USVs also have an important role in socio-sexual competency (Brudzynski, 2005; but see Ågno & Snoeren, 2015), with male rats singly housed in adolescence showing deficits in adult sexual interactions (Molenda-Figuera *et al.,* 2016) and also exhibiting reduced rates of 50kHz USV production when exposed to female conspecifics in adulthood (Inagaki *et al*., 2013). The findings in the current study that SE rats emit more 50 kHz USVs than controls therefore most plausibly indicate that, for female rats, a greater quantity of social interactions during adolescence may enhance social motivation and/or sexual competency in later-life via, or alongside, changes in vocal communication. However, further work is needed to document the behavioural correlates of 50kHz USV production to test the hypothesis that adolescent social stimulation in the current study enhances social and/or sexual competencies.

In summary, providing female rats with the opportunity to engage in social interactions, including social play behaviour, with novel partners during adolescence did not have long-term effects on later behavioural responses to novelty or glucocorticoid activity following social separation. However, females that had gained this additional social experience during adolescence

emitted higher numbers of 50kHz USVs both prior to, and following, a period of social separation compared to control groups. These findings suggest that enhanced adolescent social experience affects later social interactions in female rats and is consistent with evidence that adolescent social experience alters later vocal and social behaviour in male rats (e.g., Cumming *et al.*, 2014; Green *et al.*, 2013; Inagaki *et al.*, 2013; Seffer *et al.*, 2015). Social interactions during adolescence have been hypothesised to have long-term effects on social competence (Pellis & Pellis, 2007), defined as the ability of an individual to respond appropriately to social cues (Taborsky & Oliveira, 2012), and, in human beings, positive social interactions during adolescence potentially underpin healthy emotional and social development (Gray, 2011).

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Data Availability Statement

The data that support the findings from this study are available from the corresponding author upon reasonable request.

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Legends

Figure 1. Number of play behavior interactions occurring within SE and CE pairs during adolescent social interaction sessions (* = $p<0.05$).

Figure 2. Number of entries into the arms (a) and proportion of time spent on the arms (b) of the EPM by C, CE and SE subjects (* = $p<0.05$).

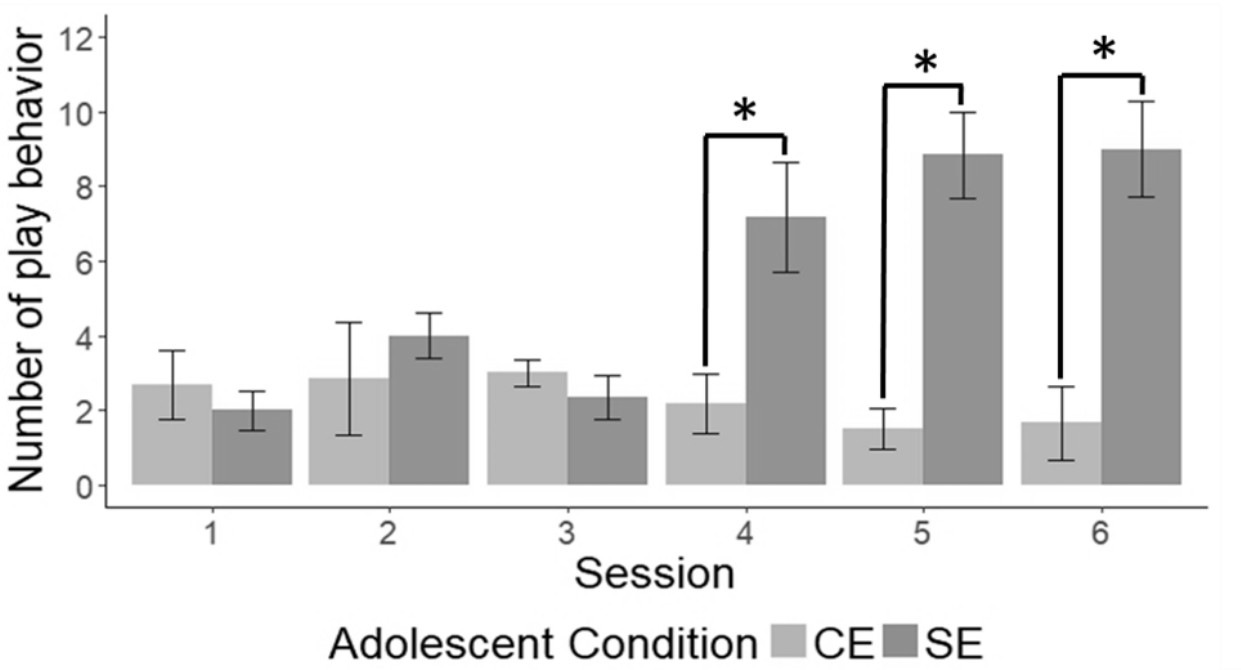
Figure 3. Number of contacts with the stimulus boxes (a) and proportion of time spent in contact with the stimulus boxes (b) in the OSN (* = $p<0.05$).

Figure 4. Total number of 50kHz USVs emitted in response to social separation and reunion averaged (mean) across test sessions (Pre = pre-separation, Sep = separation, Reu = reunion; * = $p<0.05$).

Table 1. Behavioural measures in the LDB split by adolescent condition.

Table 2. Change in 10^{-5} CORT concentration (ng/ml) scaled for creatinine concentration (mg/dl) in response to social interaction with a familiar or unfamiliar conspecific (post-interaction minus pre-interaction).

Figure 1


Number of play behavior
0
2
4
6
8
10
12
1
2
3
4
5
6
Session
*
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Adolescent Condition
CE
SE

Figure 2

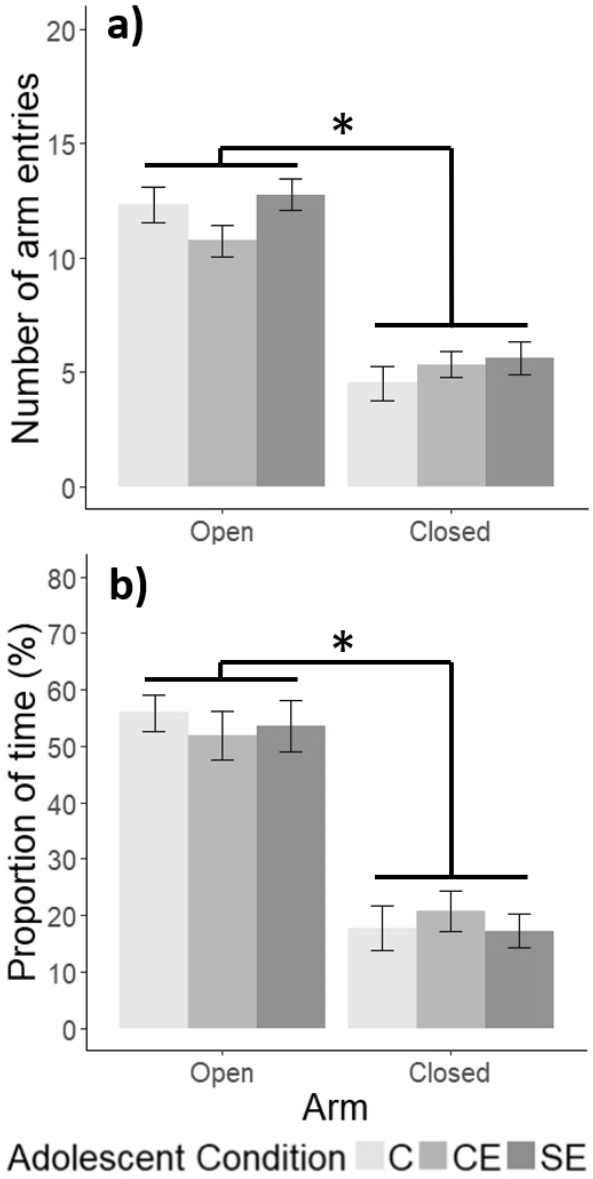
a)
Number of arm entries
20
15
10
5
0
*
Open
Closed
b)
Proportion of time (%)
80
70
60
50
40
30
20
10
0
*
Open
Closed
Arm
Adolescent Condition C CE SE

Figure 3

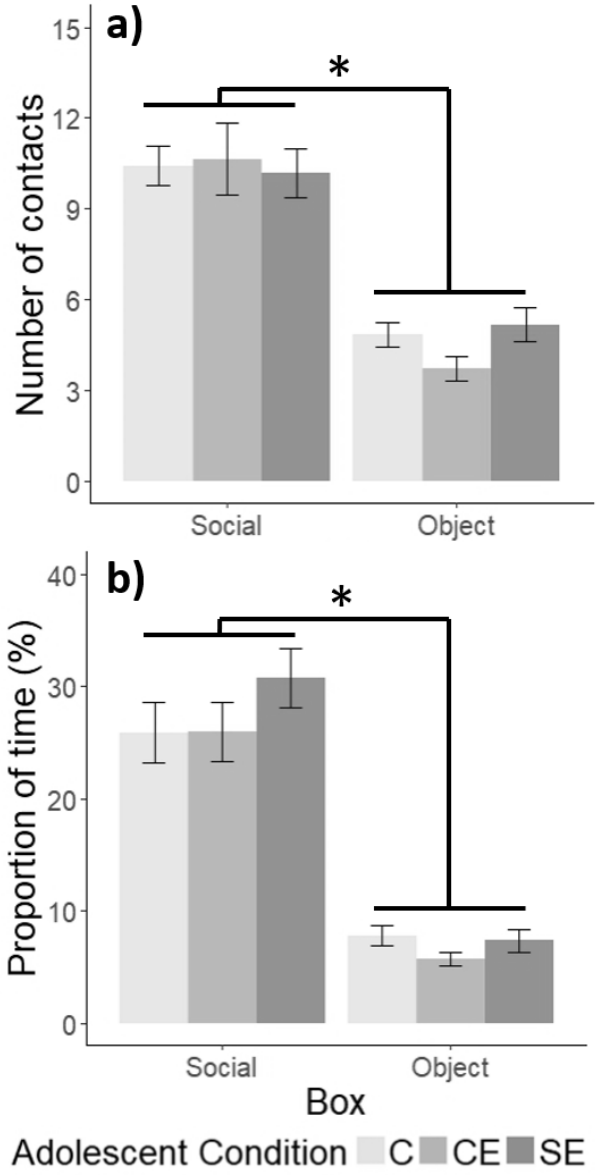
a)
Number of contacts
15
12
9
6
3
0
*
Social
Object
b)
Proportion of time (%)
40
30
20
10
0
*
Social
Object
Box
Adolescent Condition C CE SE

Figure 4

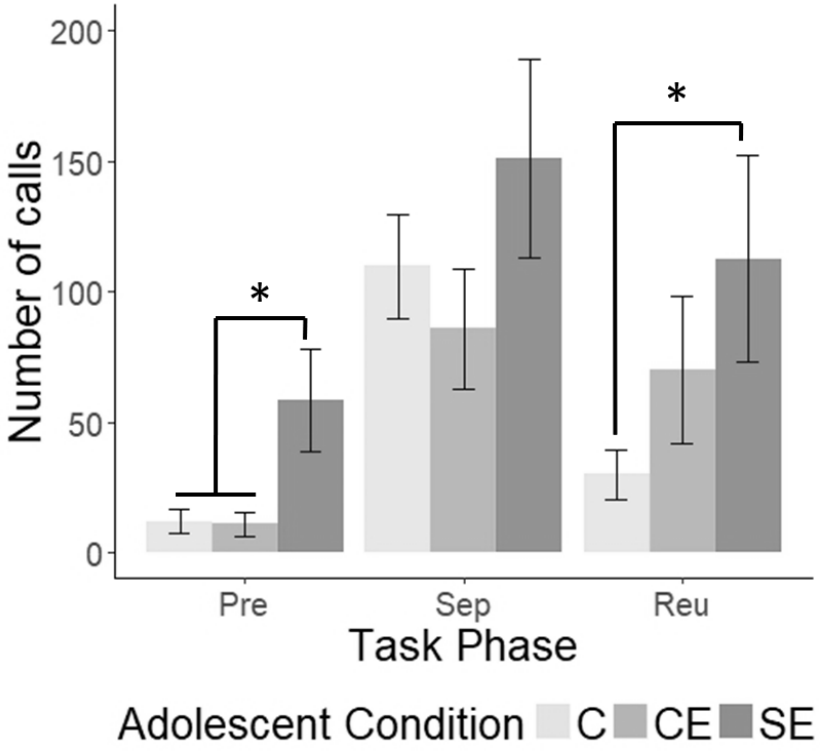
200
150
100
50
0
Number of calls
*
*
Pre
Sep
Reu
Task Phase
Adolescent Condition
C
CE
SE

Table 1

Measure	Adolescent Condition		
	C (M±SEM)	CE (M±SEM)	SE (M±SEM)
Latency to enter light compartment (sec)	52.48 (24.42)	46.8 (20.58)	49.68 (28.58)
Number of light compartment entries	3.91 (0.65)	4.00 (0.45)	4.70 (0.76)
Proportion of time in light compartment (%)	26.24 (5.19)	24.64 (4.20)	23.20 (4.69)

Table 2

Urinary Corticosterone	Adolescent Condition					
	C (M±SEM)		CE (M±SEM)		SE (M±SEM)	
	Familiar Partner	Unfamiliar Partner	Familiar Partner	Unfamiliar Partner	Familiar Partner	Unfamiliar Partner
Pre-interaction	8.52 (1.37)	10.33 (2.52)	9.93 (2.21)	14.89 (2.71)	12.85 (3.30)	12.06 (3.20)
Post-interaction	14.29 (3.46)	11.39 (2.90)	14.46 (3.46)	14.83 (3.70)	11.84 (3.38)	14.94 (3.38)
Change	5.77 (3.65)	1.06 (1.81)	4.53 (2.61)	-0.06 (4.2)	-1.01 (4.10)	2.88 (3.57)